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Positive therapeutic interaction between thiopurines and alkylating drugs in human glioma xenografts*

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Summary. We used human anaplastic glioma xenografts to evaluate the therapeutic efficacy of combinations of alkylating drugs, either 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), 1-(2-chloroethyl)-3-(2,5-dioxo-3-piperidyl)-1-nitrosourea (PCNU), or procarbazine, and thiopurines, either 6-mercaptopurine (6MP) or 6-thioguanine (6TG). Using growth delay as the endpoint in subcutaneous (s.c.) tumors and increased life span as the endpoint in intracranial (i.c.) tumors, we found that combinations of chloroethylnitrosoureas (CENUs) and thiopurines were significantly more active than either type of agent alone. In contrast, combinations of procarbazine and thiopurines were not significantly more active than procarbazine alone. The therapeutic potentiation of the CENU was greater when the latter was given on the 4th day of the thiopurine treatment cycle than when it was given on the 1st day. Characterization of the interaction between CENUs and thiopurines also revealed a supraadditive therapeutic response at higher BCNU doses in combination with 6TG. Interaction between the nitrosoureas and the thiopurines probably occurs in the guanine base of tumor DNA and has important therapeutic implications.

Introduction

The nitrosoureas are the most active drugs in the treatment of anaplastic gliomas. Both 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) and 1-(2-chloroethyl)-3-(2,6-dioxo-3-piperidyl)-1-nitrosourea (PCNU) have produced significant responses in patients with these diseases [16, 23], and nitrosoureas are the most consistently active agents in the treatment of experimental gliomas [19]. Procarbazine, a methylating agent, has also shown clinical activity [15]. However, the majority of patients with gliomas do not respond to therapy with these alkylating drugs, and numerous attempts to enhance the effect of the nitrosoureas by using bone-marrow-ablative doses, giving the drugs by other routes, or combining them with other cytotoxic drugs have been only marginally successful. The thiopurines 6-mercaptopurine (6MP) and 6-thioguanine (6TG) are active in the treatment of some human neoplasms, primarily leukemias, and probably exert their cytotoxic effects by direct incorporation of the modified bases into DNA. Since the nitrosoureas and procarbazine produce cytotoxic lesions on guanine in DNA, there is potential for their interaction with thiopurines. We report the results of experiments using human glioma xenografts in athymic mice to evaluate the therapeutic interaction of alkylating drugs with thiopurines.

Materials and methods

Animals. Homozygous adult nu/nu BALB/c athymic mice derived from an independent breeding colony at Duke University were used for these experiments.

Drugs. BCNU (carmustine; Bristol Laboratories, Evansville, Ind.) was purchased commercially. PCNU was supplied by the Division of Cancer Treatment of the National Cancer Institute. Procarbazine was supplied by Hoffman LaRoche, Inc. (Nutley, N.J.). Burroughs-Wellcome Co. (Research Triangle Park, N.C.) supplied 6MP and 6TG. All drugs were given by intraperitoneal (i.p.) injection in a volume of 30 ml/m^2 (approximately 0.2 ml/animal). BCNU was dissolved in ethanol (100 mg/3 ml) and diluted in normal saline; PCNU was dissolved in dimethylsulfoxide (DMSO, 16.5 mg/ml) and diluted in 5% dextrose; procarbazine was dissolved in normal saline; 6-MP and 6-TG were dissolved in 1 *N* NaOH (0.1 mg/ml) and diluted in 0.2 M sodium phosphate buffer (pH 8). BCNU, PCNU, and procarbazine were given in a single dose on either day 1 or day 4 of the treatment schedule. The thiopurines were given on a daily ×4 schedule. BCNU and procarbazine were given at doses that were lethal for 10% of the mice (LD_{10}) as determined in our colony (75 and 2,437 mg/m^2, respectively); PCNU was given at either 10% (in the 6MP experiment) or 20% (in the 6TG experiment) of its LD_{10} (8 and 16 mg/m^2, respectively); 6MP and 6TG were given at their LD_{10} (297

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and 24.5 mg/m^2 per dose, respectively). Lower doses of PCNU were used because it is a highly effective agent against D-54 MG, producing cures at doses as low as 50% of the LD_{10} [21]; therefore, therapeutic potentiation could be detected only at lower doses.

Drug infusions. Alzet mini-osmotic pumps (Model 2001: mean fill volume, 210 µl; mean pumping rate, 0..96 µl/h × 7 days) were purchased from the Alza Corp. (Palo Alto, Calif.). The osmotic pumps were filled with either drug vehicle or 6TG (approximately 64% of the LD_{10}) and implanted s.c. in the flank opposite the tumor when the median tumor volume of all animals in the experiment had exceeded 200 mm^3.

Tumor lines. D-54 MG is the Duke University subline of A-172 [2, 14]. It was derived from a human anaplastic astrocytoma and has been extensively described [2, 3, 25], and its sensitivity to numerous chemotherapeutic agents in athymic mice has been determined [21]. It is deficient in O^6-alkylguanine-DNA alkyltransferase (GATase) and is sensitive to nitrosoureas and procarbazine. Experiments were done with this line between the 10th and the 20th animal-passage levels. Its s.c. volume-doubling time is 2–2.5 days. N-1520 is a human brain-tumor xenograft that was established in athymic mice at Duke University after direct transplantation from a resected cerebral glioblastoma. It is also deficient in GATase and is highly sensitive to nitrosoureas and procarbazine. The treatment experiment reported herein was done at the 34th animal-passage level. The s.c. volume-doubling time for this line is 3.4 days.

Treatment experiments. The methodology of treatment experiments in athymic mice has been repeatedly described [20]. Briefly, for treatment of s.c. tumors, 50 µl tumor suspension was injected into the right flank of 40–50 animals, the tumors were measured twice weekly with calipers, and when the median tumor volume of all animals had exceeded 200 mm^3 (day 7 or 8 with D-54 MG and day 14 with N-1520), animals were divided into groups of 8–10 such that the average tumor volumes among the groups were not significantly different. Treatment began on that day (treatment day 1), and tumors were serially measured with calipers twice weekly until their volumes exceded 8 times those measured on the 1st treatment day. Tumor volume was estimated by the formula: volume = (length^2 × width)/2, where length >width. At volumes of <2.5 cm^3, growth is linear and central necrosis is minimal. For treatment of i.c. tumors, 5 µl tumor suspension that contained 50% methylcellulose was injected through a 27-gauge needle at a depth of 4 mm into the right cerebral hemisphere. Treatment began on the 11th day after i.c. tumor implantation (treatment day 1), and animals were followed until their death or until day 60 after implantation.

Evaluation of response. Therapeutic efficacy was measured in two ways in the s.c. tumor experiments. First, the number of days required for each tumor to reach a volume 8 times that measured at the initiation of treatment was determined. These values were compared among groups by the Wilcoxon rank-sum test. Growth delay, or T-C, was the difference between the median value for the treatment group and that for the control group. Second, the number of tumor regressions in each group was determined, and the values were compared by the Fisher exact test. Tumor regression was defined as any posttreatment tumor volume that was less than the volume measured at the time of alkylator administration. In the i.c. tumor experiments, the day of death of each animal was recorded and these values were compared among the groups by the Wilcoxon rank-sum test.

For the evaluation of drug interaction, the method of Deen and Williams [9] was used. Isobolograms were generated for the case in which the dose of one agent is held constant. Envelopes of additive effect for different doses of the variable agent are produced using this method. Dose-response curves for each agent alone were initially determined. The envelope of additivity was generated from a series of isoeffect levels that had been derived from the complete dose-response curves for each single agent. Combinations that produce a level of effect that falls within the envelope of mode I and II determinations are considered to be additive in effect; combinations whose level of effect falls above the envelope are supraadditive; and combinations whose level of effect fall below the envelope are subadditive [1].

Results

The growth delay produced by continuous s.c. administration of 5TG (approximately 64% of the 4-day total LD_{10} dose) was 1.1 days. Daily i.p. injections of 67% of the LD_{10} given for 4 days produced a growth delay of 1.4 days. The growth delays obtained in both treatment groups were significant in comparison with their respective control values (P <0.05 in both cases). However, the difference in growth delay produced by the two routes of

Table 1. Treatment of s.c. D-54 MG in athymic mice with alkylating agents and thiopurines

Treatment	T-C	P-value vs control	P-value vs combination	P-value vs thiopurine	TR
BCNU day 1	14.7	0.0001	0.0016	0.0001	1/10
6MP days 1–4	3	0.0001	0.0001	–	1/9
BCNU day 4, 6MP days 1–4	25	0.0001	–	–	6/9
PCNU day 1	4.3	0.0001	0.0001	0.4531	0/10
6MP days 1–4	4.4	0.0001	0.0001	–	0/10
PCNU day 4, 6MP days 1–4	10.8	0.0001	–	–	4/10
Procarbazine day 1	18.1	0.0001	0.094	0.0001	1/8
6MP days 1–4	1.4	0.0024	0.0001	–	0/8
Procarbazine day 4, 6MP days 1–4	19.9	0.0001	–	–	0/8
BCNU day 1	11.9	0.0001	0.0005	0.0001	2/10
6TG days 1–4	3.9	0.0001	0.0001	–	0/10
BCNU day 4, 6TG days 1–4	19.1	0.0001	–	–	8/10
PCNU day 1	13.7	0.0001	0.0001	0.0001	0/8
6TG days 1–4	2.9	0.0001	0.0001	–	0/8
PCNU day 4, 6TG days 1–4	21.2	0.0001	–	–	6/8
Procarbazine day 1	18.4	0.0001	0.1241	0.0001	3/8
6TG days 1–4	3.2	0.0002	0.0001	–	0/8
Procarbazine day 4, 6TG days 1–4	22.2	0.0001	–	–	7/8

T-C, Growth delay in days (see Materials and methods); TR, tumor regressions (see Materials and methods); drug doses (mg/m^2 per day i.p.): BCNU, 75; 6-MP, 297; PCNU, 8 in the 6MP experiment and 16 in the 6TG experiment; procarbazine, 2,437; 6TG, 24.5

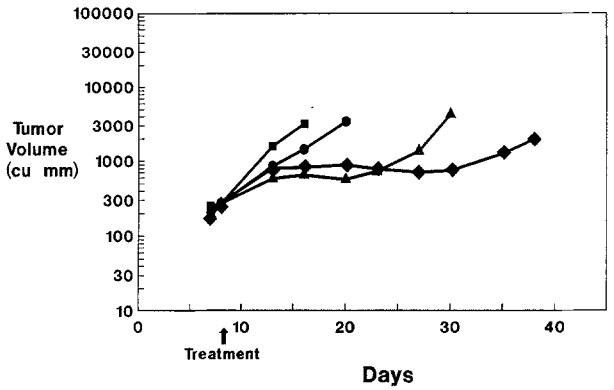

Fig. 1. Treatment of s.c. D-54 MG in athymic mice with BCNU (▲), 6MP (●), and BCNU + 6MP (◆). Treatment began on day 8 after tumor implantation (see Materials and methods for doses and schedules). Each point represents the median tumor volume in 8–10 animals. ■, controls

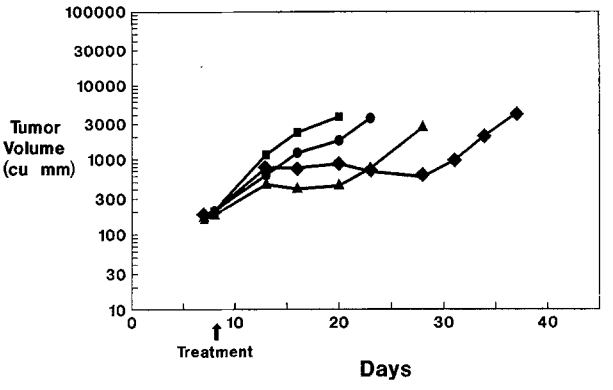

Fig. 2. Treatment of s.c. D-54 MG in athymic mice with BCNU (▲), 6TG (●), and BCNU + 6TG (◆). Treatment began on day 8 after tumor implantation (see Materials and methods for doses and schedules). Each point represents the median tumor volume in 8–10 animals. ■, controls

administration of 6TG was not statistically significant ($P = 0.20$). The daily ×4 i.p. schedule was used for all drug-interaction experiments.

Table 1 lists the results of six experiments carried out on D-54 MG using the various combinations of either BCNU, PCNU, or procarbazine and either 6MP or 6TG. The median time required for tumors to reach a volume 8 times that measured at the initiation of treatment in the six control groups ranged from 13.1 to 14.8 days. No tumor regressions occurred in any of the control groups.

Growth delay was significant ($P < 0.01$) in all treatment groups in comparison with controls. The growth delay produced by the four nitrosourea + thiopurine combinations was significantly greater than that produced by the nitrosourea alone ($P < 0.01$ in all cases). Figures 1 and 2 depict the enhanced growth delays produced by combination treatment with BCNU + thiopurine as compared with BCNU or thiopurine alone. Furthermore, the numbers of tumor regressions produced by the nitrosourea + thiopurine combinations (BCNU + 6MP, 6/9; PCNU + 6MP, 4/10; BCNU + 6TG, 8/10; PCNU + 6TG, 6/8) were significantly greater than those produced by the nitrosourea alone ($P < 0.05$ in each case). On the other hand, the growth delay produced by the two procarbazine + thiopurine combinations was not significantly greater than that produced by procarbazine alone, although the procarbazine + 6TG combination produced more tumor regressions (7/8 vs 3/8 for procarbazine alone).

Median survival values in the first i.c. experiment were: control, 24.5 days; BCNU on day 4, 41.5 days; 6TG on days 1–4, 26 days; and BCNU on day 4 + 6TG on days 1–4, 51 days (Table 2). Both BCNU alone and BCNU + 6TG produced significant increases in life span in comparison with control values ($P < 0.01$). Despite its lack of efficacy as a single agent, 6TG significantly potentiated the effect of BCNU. There were no survivors at 60 days in the control or 6TG groups, but 1/12 mice treated with BCNU alone and 3/12 animals treated with BCNU + 6TG were alive at 60 days (Fig. 3).

For evaluation of the interaction between the thiopurines and the CENUs, tumor growth-delay studies at multiple dose levels were done using 6TG alone, BCNU alone, and the two drugs in combination (Table 3). Both 6TG alone and BCNU alone produced increasing delays in tumor growth with increasing drug dose, and BCNU was more effective than 6TG. Figure 4 depicts an isobologram showing the envelope of additivity for the 6TG (at its LD_{10}) + BCNU ($0.25–0.75 \times LD_{10}$) combination. The growth delays were subadditive at lower doses of BCNU but were supraadditive at higher BCNU doses.

Median times from implantation to the achievement of a tumor volume 8 times the initial value (treatment day 1) in an s.c. experiment were: (a) BCNU on day 1, 21.3 days; (b) BCNU on day 1 + 6TG on days 1–4, 25.8 days; (c) BCNU on day 4, 16.3 days; and (d) BCNU on day 4+6TG on days 1–4, 25.6 days. The difference between

Table 2. Treatment of i.c. D-54 MG in athymic mice with BCNU and 6TG

Treatment	Animals *N*	Survival: median, range (days)	T/C	Number of 60-day survivors (%)
Control	12	24.5, 15–33	–	0 (0)
BCNU day 4	12	41.5, 19–60+	1.70*	1 (8)
6TG days 1–4	13	26, 12–36	1.10 (NS)	0 (0)
BCNU day 4 G 6TG days 1–4	12	51, 21–60+	2.10*	3 (25)

* $P < 0.05$ vs control value

T/C, median survival of treated animals/ median survival of control animals; NS, not significant; Drug doses: BCNU, 75 mg/m^2 ×1; 6TG, 24.5 mg/m^2 per day

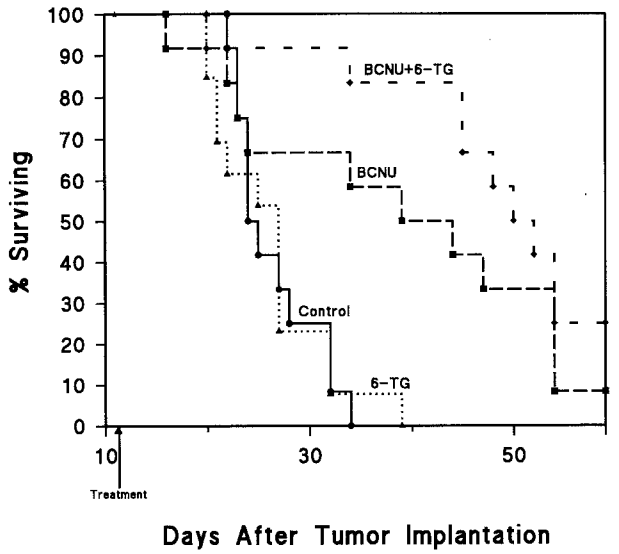

Fig. 3. Life-span distribution of animals bearing i.c. D-54 MG tumors after treatment beginning on day 11 after tumor implantation. Treatment was either drug vehicle (control), 6TG on days 11–14, BCNU on day 14, or 6TG on days 11–14 + BCNU on day 14. Drug doses: 6TG, 24.5 mg/m^2 per day, BCNU, 75 mg/m^2

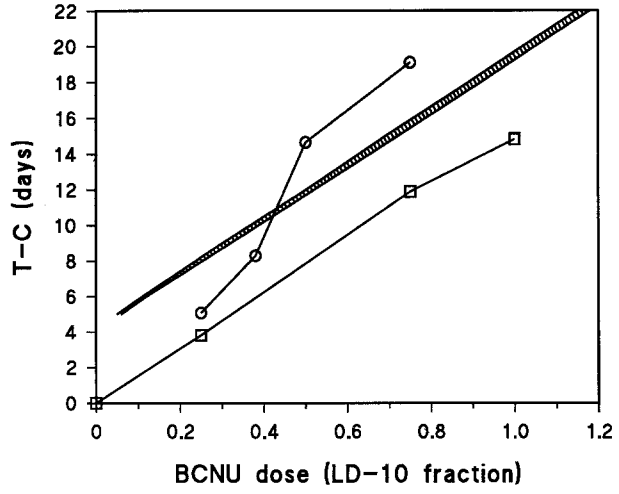

Fig. 4. Isobologram for the treatment of s.c. D-54 MG tumors with 6TG (24.5 mg/m^2) on days 1–4 and a range of BCNU doses on day 4. *Squares* represent the dose-response curve for BCNU alone. The *shaded region* is the envelope of additivity for the combination treatment. *Circles* indicate the dose-response curve for the combination treatment. Response is expressed as growth delay (*T-C*) in days

groups c and d (9.3 days) was significantly greater than that between groups a and b (4.5 days, $P = 0.046$; Table 4). Indicating that the effect of BCNU is potentiated to a greater degree by prior administration of 6TG. Furthermore, although the growth delay produced by BCNU on day 4 was less than that produced on day 1, as would be expected, the addition of 6TG rendered the BCNU treatment on day 4 equal to or better than that on day 1.

For examination of the schedule dependence of BCNU administration in i.c. tumors, BCNU was given on day 1 in a second i.c. experiment. The differences in median survival between the treatment and control groups were: (a) BCNU on day 1, 30 days; (b) BCNU on day 1 + 6TG on days 1–4, 35 days; (c) BCNU on day 4, 17 days; and (d) BCNU on day 4 + 6TG on days 1–4, 26.5 days. The combination treatment produced a 16.7% increase in median survival in comparison with BCNU given alone on day 1. In contrast, the combination treatment produced a 55.9% increase in median survival in comparison with BCNU given a alone on day 4, supporting the hypothesis that greater potentiation of the efficacy of BCNU is obtained by prior thiopurine administration. In addition, comparison of combination treatment with administration of BCNU alone revealed that BCNU given on day 4 after 6TG enhanced the percentage of 60-day survivors to a greater degree than did BCNU given on day 1 followed by 6TG (Table 5).

Table 3. Growth delay of s.c. D-54 MG produced by 6TG, BCNU, and the two-drug combination

Drug	dose (fraction of LD_{10})	T-C (days)
6TG days 1–4	0.67	1.4
	1	3.9
	1.33	6.3
BCNU day 1	0.25	3.8
	0.75	11.9
	1	14.8
BCNU day 4, 6TG days 1–4	0.25, 1	5.1
	0.25, 1	6.3
	0.38, 1	8.3
	0.5, 1	14.7
	0.75, 1	19.1

Tumor-growth delays produced against s.c. N-1520 were: 6TG on days 1–4, 6.2 days; BCNU on day 4, 27.6 days; and BCNU on day 4 + 6TG on days 1–4, 36.4 days. Growth delays were significant in all treatment groups as compared with controls ($P < 0.01$). Again, the growth delay produced by the BCNU + 6TG combination was substantially greater than that produced by either drug alone and was greater than the sum of the individual delays in growth.

Discussion

Thiopurine-induced cytotoxicity likely depends on the incorporation of the thiopurine into DNA [17]. Zimm et al. [27] have presented preliminary evidence that once a biologically active concentration of 6MP has been attained, the duration of exposure becomes an important determinant of cytotoxicity. However, the results of our comparison of a continuous 7-day infuson of 6TG vs daily bolus i.p. injections ×4 revealed no significant difference in tumor growth delay; consequently, the thiopurines were given by daily bolus injections for evaluation of their interaction with alkylating drugs in these experiments.

Positive therapeutic interaction between the CENUs and the thiopurines has been described by other investiga-

Table 4. Schedule dependence of the treatment efficacy of BCNU and BCNU + 6TG against s.c. D-54 MG

Treatment	Median time to 8× day 1 tumor volume (days)	% Increase of combination vs BCNU alone
BCNU day 1	21.3	–
BCNU day 1, 6TG days 1–4	25.8	21.1
BCNU day 4	16.3	–
BCNU day 4, 6TG days 1–4	25.6	57.1

Table 5. Schedule dependence of the treatment efficacy of BCNU and BCNU + 6TG against i.c. D-54 MG

Treatment	Survival increase over control: median, range (days)	% Increase of combination vs BCNU alone	% 60-day survivors
BCNU day 1	30, 0–45	–	25
BCNU day 1, 6TG days 1–4	35, 0–42	16.7	31
BCNU day 4	17, 0–44	–	8
BCNU day 4, 6TG days 1–4	26.5, 0–80	55.9	25

Median control survival was 22 days (range, 15–33 days) with no 60-day survivors. There were 12–13 animals in each treatment group

tors. Fujimoto and Ogawa [11] and Fujimoto and co-workers [12] reported that the combination of 6TG with 3-[(4-amino-2-methyl-5-pyrimidinyl)methyl]-1-(2-chloroethyl)-1-nitrosourea (ACNU) produced synergistic therapeutic and cytotoxic effects against L-1210 leukemia. Schabel et al. [18] observed enhancement of the cytotoxicity of PCNU by 6TG against i.c. L-1210, as the number of "cures" was increased from none following treatment with PCNU or 6TG alone to "60%–100%" following combination treatment. More recently, Bodell et al. [5] found that 6TG potentiated the cytotoxic effect of BCNU on rat 9L gliosarcoma cells in culture. The combination also increased the number of sister chromatid exchanges and DNA cross-links formed. Our data confirm and extend these observations by demonstrating that the therapeutic effect of the CENUs, BCNU and PCNU, against s.c. human glioma xenografts was significantly increased by the addition of a thiopurine, either 6MP or 6TG. However, when procarbazine was the alkylating agent, its therapeutic effect was not significantly enhanced by either of the thiopurines.

The enhanced survival of animals with i.c. human glioma xenografts that were treated with BCNU and 6TG paralleled the enhanced delay in tumor growth observed in s.c. xenografts. In addition, the schedule dependency of the drug interaction was similar in both i.c. and s.c. tumors. Indeed, the additional percentages of increase in median survival produced by the combination treatment relative to BCNU alone closely resembled the additional increases in growth delay produced by the combination treatment in s.c. tumors, for both day-1 and day-4 BCNU administration (Table 4). Thus, the use of s.c. human glioma xenografts and the measurement of their growth delay proved to be representative of drug interaction in the brain. This is particularly striking because the enhancement of the effect of BCNU by a thiopurine was observed against human glioma xenografts in the brain despite the lack of an independent effect of the thiopurine.

Initially we examined the potentiation simply in terms of a significant difference in growth delay produced by combination treatment in comparison with single-agent treatment, a definition of synergism suggested by Vendetti and Goldin [24]. However, a clearly supraadditive growth delay produced by the combination treatment in comparison with the sum of the effects of the single agents may also be viewed as a "synergistic" therapeutic response and would imply pharmacologic or biochemical interaction [1]. To define more accurately the interaction between the nitrosoureas and the thiopurines, we used an isobolar method that entails the construction of an additivity envelope that represents, in essence, the confidence limits for the summation of effects [1].

Interestingly, we found that the combination of BCNU and 6TG produced a supraadditive therapeutic effect only at the higher BCNU doses. A similar observation of the dose dependency of such interaction has been made by Fujimoto and Ogawa [11] and Fujimoto et al. [12], who reported that the synergistic effect in vivo and in vitro was obtained over a narrow range of relatively high ACNU concentrations.

The cytotoxicity of the CENUs is thought to be caused by chloroethylation at the O^6-position of guanine in DNA and the subsequent formation of lethal DNA interstrand cross-links. Chloroethyl adducts at the O^6-position of guanine in DNA are repaired by the enzyme O^6-alkylguanine-DNA alkyltransferase (GATase) [8], and in some systems, an inverse relationship between the level of this repair enzyme and CENU-induced cytotoxicity has been found [6, 7, 22]. Although the mechanism of interaction between the CENUs and the thiopurines is not known, Bodell [4] has presented evidence that DNA into which 6TG has been incorporated is more susceptible to S^6-alkylation than to O^6-alkylation, which would result in the increased formation of S^6-(2-chloroethyl)-6-thioguanine adducts. Furthermore, S^6-alkylguanine adducts do not appear to be repaired by GATase [26].

One could hypothesize that the enhancement of CENU-induced cytotoxicity results from the increased number of irreparable DNA adducts; that is, the thiopurine is initially incorporated into DNA, and subsequent exposure to a CENU produces chloroethyl adducts that are not repairable by GATase and lead to an increased number of DNA interstrand cross-links. The schedule dependency of the interaction supports this hypothesis. Thus, interference with the efficiency of the GATase repair system is a possible way of overcoming resistance to CENUs or of enhancing the sensitivity of a tumor line to these agents. Yet, whereas a number of studies have examined depletion of the GATase enzyme as a means of interfering with this repair system and potentiating CENU cytotoxicity [10, 13], the mechanism of enhancement proposed herein suggests a process in which the repair system is avoided rather than inactivated. Alternatively, the thiopurine could be directly inhibiting the repair enzyme, but this seems unlikely because of the specificity of GATase for O^6-alkyl lesions. We also found that GATase activity in mouse liver is not affected by exposure to 6MP (data not shown).

Surprisingly, similar therapeutic interaction was not seen between procarbazine and the thiopurines. Procarbazine acts as a methylating agent, and perhaps an increase in the number of cross-links after treatment with a CENU and thiopurine results in more cytotoxicity than an equivalent increase in the number of methyl adducts after treatment with procarbazine and a thiopurine. Furthermore, the dose dependency of the supraadditive effect found in the present study suggests a possible "threshold" number of chloroethyl adducts or cross-links necessary for a synergistic or supraadditive therapeutic response. Resolution of this problem requires quantitative analysis of the individual thiol adducts in DNA. Whatever the mechanism of the interaction, our data indicate that the therapeutic effect of the CENUs can be enhanced by prior treatment with thiopurines and that this effect can be seen in intracerebral tumors. Clinical trials to determine whether these data can be successfully applied to patients with anaplastic gliomas are under way.

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